The role of K antigens as virulence factors in *Klebsiella*

A. M. SIMOONS-SMIT, A. M. J. J. VERWEIJ-VAN VUGHT and D. M. MACLAREN

Research Group for Commercial Infections, Departments of Medical and Oral Microbiology, Schools of Medicine and Dentistry, Vrije Universiteit, 1007 MC Amsterdam, The Netherlands

Summary. The importance of K antigen of *Klebsiella* as a virulence factor was studied in nine pairs of K⁺ and K⁻ strains, each pair isogenic apart from the presence of K antigen. Loss of K antigen by nine K⁺ strains resulted in the reduced virulence of their K⁻ variants in a mouse-skin model. This reduced virulence of K⁻ strains for mice may be explained in all strains by a higher degree of phagocytosis as measured by chemiluminescence response of human polymorphonuclear leukocytes (PMNL) and in most strains by enhanced killing by either human PMNL or human serum or both. Although the protective role of the K antigen in serum-induced killing and killing by PMNL was generally evident, our results also suggested that other virulence factors were sometimes involved.

Introduction

Capsular antigens of *Klebsiella* have been implicated as important virulence factors in the pathogenesis of experimental klebsiella infections. In the past Toennissen (1914) and Baerthlein (1918) demonstrated that capsule formation by Friedländer's bacillus was associated with virulence in laboratory animals and Edwards (1928) showed that extensive capsule production by *Klebsiella* was necessary to produce metritis in mares. More recently, the relationship between the size of capsule of *Klebsiella pneumoniae sensu lato* and pathogenicity in experimental lobar pneumonia, in an intra-peritoneal model, or in a burn wound-sepsis model, have been shown with variants of strains of capsular serotypes 1 and 2 (Ehrenworth and Baer, 1956; Domenico *et al.*, 1982; Cryz *et al.*, 1984). Association between pathogenicity and capsule size has been studied in several other bacterial species, e.g., *Escherichia coli* (Glynn *et al.*, 1971; McCabe *et al.*, 1975; Verweij-van Vught *et al.*, 1983), pneumococcus (MacLeod and Krauss, 1950) and *Staphylococcus aureus* (Melly *et al.*, 1974). One mechanism whereby the increased virulence of encapsulated bacteria might be explained is the ability of the capsule or K antigen to prevent the killing of bacteria by serum and their phagocytosis by polymorphonuclear leukocytes (PMNL) as has been shown in particular for *E. coli* (Howard and Glynn, 1971; Rottini *et al.*, 1975; Van Dijk *et al.*, 1979; Verweij-van Vught *et al.*, 1984). Although the

degree of encapsulation has been related to virulence in experimental klebsiella infections, the exact role of these K antigens in the pathogenesis of klebsiella infections has not been established.

In a previous study in which the virulence of clinical isolates of *Klebsiella* was examined in a mouse-skin model (Simoons-Smit *et al.*, 1984), strains of capsular types K1, K2, K4 and K5 were of significantly greater virulence than strains of capsular type K6 and serotypes higher than 6. In the present study, the role of the K antigens in the virulence of *Klebsiella* was investigated by comparing capsulate (K⁺) and non-capsulate (K⁻), but otherwise isogenic, pairs of strains in a mouse-skin model and by observing their susceptibility to killing by serum and their degree of phagocytosis by PMNL.

Materials and methods

Bacterial strains

Nine strains of *K. pneumoniae* of capsular types K1 (6 strains), K2 (1) and K5 (2), previously shown to be virulent in our mouse model (Simoons-Smit *et al.*, 1984), were used in this study. Strains were biotyped and serotyped by previously described methods (Simoons-Smit *et al.*, 1984). Antibiotic sensitivity was tested by determination of minimum inhibitory concentrations in microtitration plates (MIC-2000 Dynatech).

Detection of K⁻ mutants

K⁻ mutant bacteria were detected as sectors of colonies

with a slightly more translucent appearance on nutrient agar. After subculture of these sectors on nutrient agar, single colonies were subcultured on Worfel-Ferguson Agar before confirming the absence of K antigen in capsule-swelling tests made with undiluted homologous antiserum. Mutant bacteria were biotyped and their antibiotic-susceptibility patterns determined.

Bacterial suspensions

Nutrient-broth cultures of strains in the logarithmic phase of growth were harvested by centrifugation, resuspended in phosphate-buffered saline (pH 7.4) and stored overnight at 4°C. Viable counts were determined by plating serial dilutions of the suspensions on nutrient agar. In all experiments, bacterial suspensions were diluted to $c. 1 \times 10^8$ cfu/ml.

Virulence test

Virulence for mice was tested as described previously (Simoons-Smit *et al.*, 1984). Thus, 10^7 cfu were injected subcutaneously in the back of a mouse (2 injections per mouse) and the viable counts of skin lesions estimated after 24 h by the method of Maskell (1981). The growth index of 5-mm parallel cuts at 4-mm intervals in the lesions, after plating of these cuts in a standardised way, was graded as: 8 (confluent growth), 4 (semi-confluent growth), 2 (≥ 20 colonies), 1 (< 20 colonies) and 0 (no growth). The total growth index was determined as the sum of the mean growth indices in the centre and 4 mm from the centre of the lesion. At least two mice (four lesions) were used per strain.

Preparation of PMNL

PMNL were separated from heparinised blood from healthy volunteers as described before (Simoons-Smit *et al.*, 1985). The PMNL were resuspended in Hanks's Balanced Salts Solution (HBSS) to 5×10^6 PMNL/ml for chemiluminescence (CL) experiments. For killing experiments, the NH_4Cl treatment of PMNL for lysis of residual red cells was omitted and the PMNL were resuspended, after washing, to 1×10^7 PMNL/ml.

Chemiluminescence (CL)

The induction of CL of PMNL was measured as described before (Simoons-Smit *et al.*, 1985).

Serum resistance

The bactericidal effect of serum was estimated as follows: 0.1 ml of bacterial suspension (1×10^8 bacteria/ml), 0.1 ml of pooled normal human serum (NHS) and 0.8 ml of HBSS in polystyrene vials were incubated with shaking in a waterbath at 37°C. Control tubes with heat-inactivated (56°C for 30 min) human serum (HIS) were included. Viable bacteria were estimated after incubation

for 30, 60 and 90 min. The degree of serum resistance was graded as: $- = \geq 50\%$; $+ = 11-50\%$; $++ = 1-10\%$; $+++ = < 1\%$ survival of the inoculum.

Killing by PMNL

Killing experiments by human PMNL were performed as follows: 0.3 ml of HBSS, 0.1 ml of NHS, 0.5 ml of PMNL (1×10^7 PMNL/ml) and 0.1 ml of bacterial suspension (1×10^8 bacteria/ml), giving a bacterium: leukocyte ratio of 2:1, were mixed in polystyrene vials and incubated with shaking in a waterbath at 37°C. Samples (0.1 ml) taken after 30, 60 and 90 min were added to 9.9 ml of distilled water at 0°C to lyse the PMNL without killing bacteria. Viable counts were determined by plating serial dilutions on nutrient agar.

Results

Isolation of K^- mutant strains of *Klebsiella*

From the nine *Klebsiella* strains, K^- mutant bacteria were isolated without mutagenic treatment. None of the K^- isolates showed a positive capsule-swelling reaction with undiluted homologous antiserum whereas the capsulate parent strain gave positive capsule-swelling reactions with homologous antiserum at its working dilution. Differences in biotype or antibiotic-sensitivity pattern of parent and mutant strains were not observed.

Fig. 1. Kinetics of chemiluminescence of PMNL activated by K^+ and K^- variants of *Klebsiella* strain KP1 in the presence of normal human serum (10% NHS), or heat-inactivated human serum (HIS), or in the absence of normal human serum (0% NHS).

Virulence for mice

The virulence of the K⁺ and K⁻ strains was tested in a mouse model. Macroscopic examination revealed great differences in the lesions produced after 24 h by K⁺ and K⁻ strains. Thus, all K⁺ strains produced small abscesses with pus but without marked necrosis, whereas the K⁻ strains produced mild induration only. The mean total growth indices of K⁻ strains were much less than those of K⁺ strains (table) and were similar to those previously reported for the avirulent strains of capsular types K6 and higher. During passage through mice, K⁻ strains did not revert to the K⁺ phenotype.

Chemiluminescence

CL determinations measured the ability of K⁺ and K⁻ strains to activate PMNL metabolism in the presence of serum. The results of one such experiment with the KP1 pair of strains are presented in fig. 1. Without NHS or with HIS, the CL response was always much lower than that of the same strain

in the presence of 10% NHS indicating that complement was needed for optimal opsonisation of K⁺ and K⁻ strains. The CL responses of the K⁺ and K⁻ strains, expressed as percentages of the CL responses induced by zymosan, are given in the table. The CL response of human PMNL was always higher for K⁻ than K⁺ strains.

Serum resistance

The K⁺ and K⁻ strains were tested for their sensitivity to killing in 10% NHS and the results of one such experiment are shown (fig. 2). The results for all K⁺ and K⁻ mutant strains are given in the table. These representative results showing changes in serum resistance after incubation for 60 min were also evident at 30 and 90 min. Seven of the nine K⁺ strains were serum-resistant although a small initial killing was sometimes observed. With the loss of K antigen, five strains showed an enhanced killing in 10% NHS with a corresponding decrease in viable counts (to <1%) in the first 30-min period. For the remaining four strains, however, differences in

Table 1. Virulence, phagocytosis and killing of *Klebsiella* strains of different capsular serotypes and of their K antigen-negative mutant strains

Strain no.	Capsular serotype (K)	Virulence (mean total growth index)	Chemiluminescence*	Killing†	
				10% NHS	PMNL + 10% NHS
KP1.K ⁺	1	23.5	29.5	—	+
KP1.K ⁻	...	5.2	132.5	—	+++
KP2.K ⁺	1	14.0	51.5	—	+
KP2.K ⁻	...	6.9	181.5	—	+++
KP3.K ⁺	1	17.1	83.0	—	+
KP3.K ⁻	...	5.5	179.5	+++	NM
KP4.K ⁺	1	20.4	28.5	+	+
KP4.K ⁻	...	4.6	143.5	+++	NM
KP5.K ⁺	1	24.0	38.5	+	+
KP5.K ⁻	...	5.4	122.5	+++	NM
KP6.K ⁺	1	24.0	14.5	—	—
KP6.K ⁻	...	5.2	123.0	+++	NM
KP7.K ⁺	2	20.5	24.5	—	—
KP7.K ⁻	...	5.2	134.5	—	+++
KP8.K ⁺	5	24.0	46.0	—	—
KP8.K ⁻	...	5.4	179.5	+++	NM
KP9.K ⁺	5	22.0	74.5	—	+++
KP9.K ⁻	...	5.7	190.0	—	+++

* Percentage of value with zymosan; values are the mean of at least two experiments.

† Survival of the inoculum after incubation for 60 min was: — = >50%; + = 11–50%; ++ = 1–10%; +++ = <1%. Values are the mean of 2–6 experiments. NM = not measurable due to extreme sensitivity to killing by 10% NHS alone.

Fig. 2. Killing of K⁺ and K⁻ variants of *Klebsiella* strain KP2 by PMNL in the presence of 10% normal human serum (PMNL/NHS). Control experiments were made in the presence of 10% normal human serum (NHS) or heat-inactivated human serum (HIS) without PMNL.

serum killing between K⁺ and K⁻ strains were not observed. In control tests with 10% HIS, killing of K⁺ or K⁻ strains was not observed.

Killing by PMNL

Tests of killing by PMNL in the presence of 10% NHS were performed for all K⁺ strains and the four serum-resistant K⁻ strains and the results compared with those observed in 10% NHS alone. With the other five K⁻ strains killing by PMNL was not measurable because of their extreme sensitivity to the lethal effects of 10% NHS alone. The viable counts of K⁺ and K⁻ variants of strain KP2 after incubation with PMNL and 10% NHS, with 10% NHS alone and with 10% HIS are shown (fig. 2). The results of killing by PMNL in the presence of 10% NHS after incubation for 60 min are shown in the table for all strains tested. Again, the results representative of the changes in killing after incubation for 60 min were also evident at 30 and 90 min. There was little, or no, killing of K⁺ strains other than strain KP9. An increase in the killing by PMNL was observed for three of the four serum-resistant K⁻ strains; both the K⁺ and K⁻ variants of strain KP9 were extremely sensitive to killing by PMNL.

Discussion

The importance of capsular polysaccharide of *Klebsiella* in relation to virulence has been well

established (Ehrenworth and Baer, 1956; Hall and Humphries, 1958; Domenico *et al.*, 1982; Mizuta *et al.*, 1983; Williams *et al.*, 1983; Cryz *et al.*, 1984). Several hypotheses have been proposed for this involvement of K antigens in virulence, e.g., anti-phagocytic activity and a barrier function against non-specific host-defence systems (Cryz *et al.*, 1984).

This study was designed to investigate the influence on virulence of the K antigen of *Klebsiella* and to provide an explanation for the importance of these K antigens in infections. Strains isogenic apart from differences in K antigen were used; furthermore, K⁻ strains were isolated without mutagenic treatment so that the occurrence of multiple mutations seemed unlikely.

With the loss of K antigen, the virulence of all K⁻ mutant strains was reduced in our mouse model suggesting that K antigen is an important factor in the establishment of infection by strains of *Klebsiella* of serotypes K1, K2 and K5. Encapsulation may increase virulence by interference with phagocytosis and serum killing. All K⁻ strains showed higher stimulation of CL in PMNL than the corresponding K⁺ strains indicating better opsonisation of the K⁻ strains. The higher CL obtained with the K⁻ strains correlated well with the results of virulence when the strains were tested in our mouse model.

The interference of the K antigen with phagocytosis as measured with CL is confirmed by the results of our killing experiments with human PMNL. Encapsulated bacteria require specific anti-capsular antibodies for effective opsonisation, as has been reported for *E. coli* (Welch and Stevens, 1979; Welch *et al.*, 1979; Van Dijk *et al.*, 1981) and *Klebsiella* (Williams *et al.*, 1983). Because K antigens are generally less immunogenic than O antigens, the relative lack of anti-K antibodies in our pooled normal human serum (data not shown) may be responsible for the observed differences in opsonisation of K⁺ and K⁻ strains.

The results of serum-bactericidal tests performed with the K⁺ strains and their K⁻ derivatives indicated that K antigens also determined resistance to serum killing in at least five strains. It is known that many factors may influence the complement-mediated bactericidal action of NHS. Besides K antigen, other components of the cell envelope, some of which have been shown to be plasmid-mediated (Reynard and Beck, 1976), are of great importance. Williams *et al.* (1983) emphasised the role of both O and K antigens in determining the resistance of *K. aerogenes* to serum killing and phagocytosis. From our experiments it is not clear how far O antigens

and outer-membrane proteins contribute to the serum resistance of our serum-resistant K⁻ mutant strains. For those K⁻ derivative strains that remained serum-resistant, the K antigen was presumably not a critical factor. Because we observed differences between K⁺ and K⁻ strains in opsonisation *via* complement, it seems likely that the late components of complement did not function in these serum-resistant K⁻ strains.

From the observed differences between our isogenic K⁺ and K⁻ strains of *Klebsiella* in virulence,

serum killing, phagocytosis (as measured by CL) and killing by human PMNL, we conclude that K antigen is an important virulence factor in *Klebsiella* strains. Nevertheless, other factors may affect the resistance of some strains to killing by serum and PMNL.

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